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PURE EPITHELIAL CULTURES ON PLASTIC MEMBRANES WITHOUT TRANSPLANTING AND WITHOUT PLASMA: RAPID PREPARATION OF COLORABLE CULTURES AS SMEARS, by John Wirth and Georges Barski (Pasteur Institute. Virus Service).

Pure Epithelial Cultures and Virus Cultures

This work is of purely practical significance. As early as 1929 Andrews had stated that tissue culture had become a technic applicable to the most varied problems. We will limit ourselves here to a single application of the method, i.e., the study of ultrafiltrent virus.

The possibility of spreading virus on tissue cultures has existed for 30 years; the method has notably spread resulting from the simplification introduced by Maitland and later by Li and by Rivers. Before the work by these authors, tissue culture, while perfected by Carrel and Fischer's work, had remained too complex for widespread use. Mass culture, in simple liquid media, worked out by the above authors, has allowed prolonged in vitro cultures of very numerous viruses. This culture during the past ten years has allowed numerous observations on virus variations, which in turn have led to the preparation of strains which may be used clinically as vaccines. The great interest caused by all these works and the widespread impression that tissue culture is a remarkable weapon in the fight against virus diseases, however force us to admit that the method has not yet been completely exhausted in this field.

The data of virus cultivators is difficult to interpret and, because of this, of unequal value, because their tissues are often neither in pure culture, nor even in culture, according to the definition

accepted by Carrel. Carrel's cultures in hanging drops and in flasks are used too rarely for virus cultures today to invalidate our conclusion. Maitland's method, as applied in practice, does not employ a tissue culture, as the cells are not transplanted, but periodically renewed. The same is true of agar-tissue, developed by Zinsser and Cheever. Only the roller-tube method proposed by Carrel and applied on a wide scale by Gey and Bang, consists of a true tissue culture, and has the possibility to proliferate virus on pure cellular strains. We will study this last point, the use of pure cultures, despite the fact that pure fibroblast cultures are so easy and so fast that they have often been used to nourish certain virus experiments which we do not have to repeat here. On the other hand use of pure cultures of epithelial parenchyma has been relatively rare. The affinity of most virus to these cells, the diversity of epitheliums, their persistant autonomy in vitro, the variation of their reactions when exposed to different viruses, the wonderful possibility of histological studies offered by their cultures, are all reasons which make the lack of interest in laboratories devoted to virus studies inexplicable.

Just as the propagation of virus in vitro only assumed its full significance after the important simplifications introduced by Maitland, by Li and Rivers, thus we believe that the relative complexities of pure epithelial culture are slowing up its application to actual problems and mainly the transferal of theoretic knowledge accumulated in various laboratories to the practical domain.

Previous Applications of Pure Epithelial Cultures

As has been already stated, these applications have been rare, in comparison with work done on cultures of mixed tissues. As far as we know, only one group of authors has used pure epithelial membranes for virus cultures: Kobe and Ferdig, in 1936, used pure iridien epithelian membranes for a half a dozen transplantings of aphteus virus.

Epithelial cultures in hanging or horizontal drops have allowed the study of interaction between virus and cells. Excellent results have been obtained by scientists using this technique. Among the fifty authors who have published such works let us name Nauck, in 1936, who studied the ectromeric agent in tissue culture of pure epithelium, and who also described, in 1937, the action of the lymphogranulomatous virus on the same material. Let us remember the work done by Malamos (1938), who also cultivated the lymphogranulomatous virus on pure epithelial membranes. The psittacosis and vaccine virus, have in the same way quite often been used in the study of the action on cells of pure epithelial culture. Nauck & Robinow (1935), Bland and Robinow (1939), Bland and Canti (1935).

Techniques Used Up-to-date in Obtaining Pure Epithelial Membranes

It is helpful to recall all the techniques perfected in tissue cultures of laboratories. The classical method, that of Carrel, consists of cultivating parenchyme epithelial tissue on the surface of coagulated plasma drops. The tissuelar fragment is stuck to the surface at the

very moment of coagulation. The maneuver, sometimes delicate, is not indispensible; one may await the firm coagulation of the center and place the tissue on the plasmatic surface, on condition however to moisten the surface of the drop by the means of a glass spatulla and of a small amount of physiologic solution. It must then be cultivated in a horizontal position, in an incubator of declining heat, so as to cause condensation toward the lower end. The formation of epithelial membranes is thus facilitated. In practice, the fibroblast, penetrating the plasmatic medium, are very prolific at the beginning; it is not until the third or fourth transferral that the pure epithelial membranes develop practically exclusively. This probably stems from the fact that the explants are by now completely covered by an epithelial membrane. The age of the embryonic tissue plays a primordial role. The older the embryo the more rapidly as a consequence of transplanting the epithelium propagates. These pure epithelial membranes are therefore found on the surface of the coagulated plasma. The digestion of the plasma then appears, characteristic of epithelial tissues; the primitive epithelial membrane is perforated by holes caused by the disappearance of the liquified plasmatic support, all the membrane cells are retracted toward the periphery, forming a dense epithelial ring, which cannot be further studied histolocially, but is still viable. The continued digestion of the plasma drop, the fragment of transplanted tissue finally comes into contact with glass; from that moment, a second ring proliferation might take place, on the basis of the initial tissue and also of the peripheral ring. These membranes often become very

nice and easy to stain, situated as they are directly in contact with the glass. However there have always been irregular shapes. They do not allow quantitative analysis (Fig. 2). At this moment, the fibroblasts do not prosper at all since all fibrinous matter is lacking. This technique has been used by the Hamburg and London researchers (Mauck, Malamos, Bland, Robinow).

At present I cannot go into details about the various characteristics of culture, according to embryonic tissue, the age of the tissue, and the animal species. It should be remembered that the kidney of adult animals seems to be the only tissue from which one might obtain pure epithelial cultures in the plasmatic medium at the first transplanting.

The tissue culture directly on glass slides, without plasma, has often been described (Drew, Lewis, Fischer, Heaton, Santesson, and others). According to the opinion of the culturists themselves, it is more a case of a method of exception, inferior in its results to those obtained through plasma culture. The glass surface does not permit rapid and easy extension of the epithelial membranes necessary for an active and indefinite proliferation. Santesson, in his remarkable study on neoplastic tissue culture in the mouse reviews the various supporting evidence found in literature (agar, fibrinogen, egg albumin, colloidine, cellophane, etc.). This author has chosen plasma despite its inconveniences, for it gives by far the best results.

A third method of pure epithelial culture consists in encouraging the epithelium as opposed to the fibroblasts, by using certa in substances, known as growth differentiators, Heaton, at the time of his experiments with such products, was brought to study the conditions of a membranous epithelial extension. He repeatedly states that the liquid mediums (with or without

serum or extract) are not often used for they only allow an inferior proliferation and are more difficultly maintained than plasmatic media. On the other hand, cultures in liquid media are irregular, subject more than others to dessication through pH variations and sensitive to fat particles on the glass surface; such cultures allow no quantitative analysis. Medawar, in 1937, continuing the studies of Heaton, also chose the culture on plastic coagulum as being by far the most satisfactory (p.151). He underlines the irregularity of epithelial membranes obtained when the plasmatic digestion is intense (intestine), he states that plasma is indispensable, seeing that cultures in liquid media are variable.

Let us remember the opinion of Katzentein and Knake, who believed that all substances which diminished the superficial tension were favorable to the epithelium and inhibited the fibroblasts. In practice, application of these methods to virus culture, the diggnostic, the testing and histological studies, come against actual difficulties. The method of Carrel has absolutely constant results, the further digestion of the center, necessitates:

1. The use of plasma which frequently greatly interferes with coloring and the histological study;

2. Transplantings, always indispensable in great or small numbers, which implies a delay of from ten days to two weeks before the cultures can be used.

On the other hand, the transplanting always entails a loss by technical accidents or by infections which after three to four transplantings diminishes by the half of cultures at hand. Further, we will state that the secondary growth on glass, after the central digestion, is far from regular, seeing that it develops as the proteolytic digestion progresses.

Culture on glass slides, without plasma, does not give a growth which is abundant and energetic enough; the cellular emmigration as observed, does not allow for elimination of the explant and the continuation of the culture, by nutritive bathing of the cells having prospered on the surface of the glass (Heaton, p.294). The alimentation of tissues by liquids containing additional growth differentiators, even when it allows for rapid pure epithelial cultures, does not eliminate the convenience of plasma (Medawar, p.151) and does not allow for rapid and regular extension on the glass surface.

Sought Improvements

The study of tissuelar cultures application to the problems set by virus diseases has convinced us of the advantage in perfecting a technique allowing rapid attainment of pure epithelial cultures which would be devoid of plasma or easy to stain.

We therefore have experimented with a great number of varied culture systems:

1. In a liquid medium (thyroid extract serum), between two glass slides kept at varied distances so as to allow good oxygenation: complete failure.

2. Between, or under cellophane membranes: irregular results, always mediocre.

3. On glass slides, covered by small squares of gauze embued with:

   a. Liquid medium: failure

   b. Coagulated plasma: good proliferation, but always against the plasma, never on the glass.

4. Transfer of membranes obtained on plasma: failure

5. Tryptic digestion of the plasmatic support covered with epithelial membranes: failure.

6. Culture on glass slides covered with the thin coat of plastic matter, according to the technique of Porter, Claude and Fullam. Like these authors, we obtained a cellular immigration both fibroblastic and epithelial, but not vigorous, too poor for a sustained culture after removal of the explant.

7. Having had difficulties in separating the plastic membranes supporting the cells because of the electric microscope, we change the tehhnique of the American authors by correcting a very thin plastic membrane, formed on the surface of a physiological liquid, on glass slides. These membranes, separated from the glass by a thin layer of liquid, immediately showed themselves extremely favorable in the proliferation of the epithelium excluding the fibroblasts. Proliferation of the epithelial cells on the damp plastic membranes after two to three days was by far superior to the best results obtained on plasma with repeated transplantings.

8. From that moment we started perfecting the methods. We had great difficulty in repeating the results obtained at the beginning, as every detail proved to be of great importance. Evaporation has to be minute, aeration good, the quantitative relation between tissue and liquid must be strictly enforced. The meniscus must be concave, the pH very definite. The method finally perfected which we will describe, gives with perfect regularity large pure epithelial membranes which are very fine, confluent, in very few days, andwithout the use of plasma. After fixation with oxmic vapors, these preparations are simply washed in distilled water, dried and treated like smears. The most varied colorations and observation under immersion are then possible.

Adopted Techniques

Choice of Tissue. Here we will only described pulmonary tissue cultures, this tissue being most familiar to us.

The lungs of chicken embryos of from 15 to 20 days incubation are extracted with the usual precautions. This tissue is cut out on a microscope slide (a few drops of Thyroid liquid added) by means of special razors renewed at every sitting; it is important that the tissue be cut and not torn or squashed. The sections to be placed in culture must be small: 1/4 or 1/2 of a metal pinhead, larger fragments do not give epithelial proliferation and contain so many red corpuscles, leucocytes and macrophages, that the clarity of the epithelial image is compromised. First eliminate the greatest number of free cells possible, we let the fragments remain in a few drops of thyroid from two to three hours at room temperature.

Preparation of the Medium. This factor strikes us as having great importance. Cultures with identical techniques, but with media simply composed by varying proportions of serum, with embryonic extracts and thyroid have given no satisfactory results. The liquid resulting from centrifuging coagulated plasma with extract is not necessarily identical to a simple serum and extract mixture, we therefore chose a liquid resulting from centrifuged coagulum as being the closest to the plasmatic medium (following the other authors). It has seemed possible to us that certain extract factors are altered because of its coagulating activity, so that we added fresh extract to the liquid which we have already mentioned, the whole being diluted with thyroid.

In practice, we proceed in the following manner: an amount of extract is mixed with a certain amount of plasma (in a centrifuge tube containing glass marbles). The coagulum which results in approximately 5 minutes is agitated violently for 2 minutes, which reduces it to very small particles. The whole is centrifuged at 5000 rpm for 10 minutes. The one part extract and two parts thyroid are added to the resulting liquid transferred into a sterile tube. We add 100 units of penicillin per cc at regular intervals to this mixture. It is dangerous to use less penicillin, for this product loses its power rapidly in this medium and at 37°. Larger concentrations are harmful to tissue, not so much because of the penicillin, but because of the impurities still abundant in the commercial samples of this product. The medium is therefore ready for use and may be kept in a refrigerator for 4 days (+ 2 to + 4°). We must state that our thyroid is prepared freshly from stock solutions, before every culture mixture; the formula is classical, except for the fact that we have quadrupled the quantity of monosodium phosphate so as to counteract the alkalyzing effect of the Seitz filtration. This filtration has to be conducted under pressure so as not to allow an escape of CO_2 (Thomas). The thyroid is added to 50 mg per 100 of red phenol so as to indicate the pH. This results in our embryonic extract, and our cultures, being stained salmon pink, which not only allows a regular control of the pH, but also a rapid estimation of the quantity of liquid covering the cultures, by the more or less intense coloration of the drops.

The extract is made with an embryo 14 days old for 30 cm of thyroid. We prefer to cut up an embryo into particles the size of the head of pins

with scissors instead of mashing them with marbles or with a press, a process which gives a useful extract, but not too full of cellular debris.

Our chicken plasma is obtained by punction of the jugular with a frozen syringe and a strong needle (1 mm.). We use no anticoagulant; this plasma is well kept in paraffined and coaled (0°) tubes.

Incubation. The cultures are made in lying drops, because of the fact that the fragment of tissue must rest by its own weight on the surface where the epithelium will have to spread. The tubes will therefore have to be placed in an incubator in which the heating element is at the top. Condensation will thus be at the bottom. The surface of the Petri dishes in which the cultures will be conducted are relatively large and therefore will have to be placed where the heat is most regularly distributed, otherwise it will be inevitable to produce a relative dissecation of certain drops. For these same reasons, one must avoid taking out the cultures before the moment of observation and nutritive washing; every cooling of the boxes produces a condensation; as the surface represented by the cultures is always smaller than the surface in the Petri dishes and on the glass of the base, all condensation followed by new evaporation would dry out the cultures.

The incubation temperature which seems most favorable to us is 36°. The observations and nutritive baths are made at intervals of from 2 to 3 days.

Description of the Recipients. The cultures are made on small glass slides cut into microscope slides. These slides, numbering 6, are disposed on a large glass square of ordinary glass and are covered by half a Petri

dish. The sealing is conducted with a bent pipette and very hot paraffin (smoking). These small slides are stuck on the glass with vaseline (see below) and on them, the plastic membrane is kept in place by small square rings, made out of strong glass rods 3 mm. thick.

Preparation of the slides. The entire surface of these panes (squares of ordinary glass 12 cm. by 12 cm.) must be covered with vaseline, with the exception of 6 windows corresponding to the central sections of the 6 slides which hold the plastic membranes and cultures. This vaseline coat has 4 distinct purposes:

a. It greatly diminishes the condensation of the surface of the glass;

b. It avoids the escape through capillarity of nutritive liquids disposed on the blades;

c. It strongly holds the slides in place, facilitating later manipulations: fixation, bathings, colorings;

d. It hinders transmission of infection from one slide to another.

With very hot vaseline (smoking) and a paint brush, one may outline 6 windows with 7 strokes of the brush; in dimensions these will be 5 mm. smaller than the small glass slides. Once the vaseline has been placed, one may temporarily replace the Petri dish cover.

Confection of the plastic membranes and placing of the small slides. The plastic matter used is Formvar (American polyvinyl) [See Porter, Claude and Fullam], in a solution of 0.6% in ethylene chloride.

A Petri dish is filled to the brim with Ringer liquid strongly stained with pink coloring by the use of red phenol (0.15%). With a Pasteur pipette one places a drop of Forvar solution onto the Ringer surface to the height of 6 cm. A plastic membrane immediately forms

on the surface of the liquid. It must be approximately 5 cm. in diameter, that remains invisible except for the center, thicker and often more defective. A blade is taken with a tweezer and is slipped from the edge of the Petri dish under the membrane; the latter is thus collected on the blade while letting the most possible liquid run off, and allowing the ends of the membrane to fold under the blade. The blade is then deposited on the vaseline slide, over one of the window panes. When the 6 blades are in place, they are strongly applied against the glass with a sterile glass rod, so that the four edges are well sunken into the vaseline.

The red phenol shows up progressive acidification of the Ringer liquid which must be changed as soon as the pH drops below 7. Between membrane infection, it is good to check by the use of tweezers that no membrane fragment stays above the liquid, which would get into the way of the next forming membrane.

Placing of cultures. A small glass ring is placed on every blade. The nutritive liquid is placed in the delineated space, drop by drop, making sure that the entire plastic surface is regularly dampened without being torn. The liquid must finally reach the upper section of the ring. Four or 6 fragments of tissue are then deposited in the liquid. It is essential that these fragments: ~~of tissue are then deposited in the~~

a. Fall to the bottom of the liquid, in contact with the plastic membrane;

b. Are well centered, at equal distances one from the other and from the sides of the ring;

c. That they are not disturbed by rash movements during the drying and the transfer to the centers used.

The liquid then filters under the ring and covers the entire blade. The glass ring however retains the necessary amounts of liquid over the tissue because of capillary action. Moreover, the meniscus above the cultures is concave, which appeared to be of a certain importance. As a matter of fact, cultures without rings, covered by a sufficient quantity of liquid that was convex meniscus drops repeatedly gave us very inferior results.

Observation and Washing of Cultures. A nutritive bathing becomes necessary after three days. The observation may take place with little magnification, through the Petri dish. For greater magnification, the following procedure is necessary. The containers are cooled to room temperature for 15 minutes; during this time sterile glass slides, of smaller dimensions than thoseused for the cultures, are warmed in the incubator. Their petri containers are then unstuck and replaced by these warm panes which will rest on the glass rings. Medium magnification is thus possible, without possibility of fogging up.

So as to conduct a nutritive bathing, the cultures liquid is drawn off with a pipette and ball, then replaced by nutritive liquid described above. The containers are again dried and placed in incubators.

During this maneuver, and to obtain pure epithelial cultures, their initial tissue fragments may be delicately detached and picked off with tweezers. The newly formed epithelial membranes are abundant enough to continue proliferation after the washing.

Fixation and Coloring. The containers are opened, the liquid drawn off, then the glass rings are delicately removed with tweezers. The glass stains with the 6 blades stuck on the vaseline are then turned over into

Petri dish tops containing a few cubic centimeters of osmic acid at 1%. Two minutes of fixation are enough: the tissue fragments must in no case go brown. As soon as a pale brown coloring is attained in the tissues, fixation must be stopped. The cultures are then abundantly washed by distilled water, so as to remove all trace of a nutritive media; this is a very delicate step in the operation, since the damp plastic membranes are extremely thin and break very easily. This washing may be conducted more safely by holding the membranes in place with the glass rings. Once the washing has been completed, the cultures are placed to dry in a vertical position.

The dry preparations are extremely resistant and may be colored like ordinary smears. We like using the Giemsa coloration as it is used in hematology.

Fixation with osmic acid strongly points out the greasy residue so that it is preferable never to allow the cultures to degenerate by spacing their bathing too far apart.

It is evident that osmic acid may be replaced by fixation with an appropriate liquid.

Results

The obtained cultures have the advantage of being of great regularity they thus allow for quantitative estimates (fig. 1).

Under the first hundred cultures of pulmonary tissue effectuated through the use of this technique, we obtained 95% of excellent and regular cultures; the 5% of failure were due to 3 cultures which failed because of a tear in the plastic membrane and 2 cultures which were spoiled by the proliferation of a fungus. The growth is completely

regular, circular, and reaches an approximate diameter of 3 mm. after 2 days of culture, a diameter which attains 6 or 7 mm. 3 days after the first bathing.

When a number of tissue fragments are located at the amall distance from one another, the proliferation is rapidly confluent. In the same way, after the extraction of the explant, the central void is rapidly filled by new tissue.

After the first hours of culture, immigration of certain fibroblasts takes place; very rapidly, and before 24 hours, the epithelial proliferation takes the upper hand. In no case did we note a fibroblastic proliferation, even in the case of isolated elements, after one or two days of culture.

All the characteristics of pure epithelial membranes which proliferate on coagulated plasma or on the surface of glass resulting from plasmatic digestion may be found in the membranes cultivated by this process. Mitoses are relatively frequent, around the edges of the membrane, in inferior numbers however to the amitoses. On the edges, one may find protoplasmic extensions, and eosinophiles demonstrating the amoeboid progression of marginal cells.

General Remarks

It is evident that all manipulations are conducted with sterility precautions used during tissue culture (work in glass cages, etc.).

Paraffin used for sealing, as well as vaseline, must be applied when extremely hot.

Experience consisting of approximately 1000 cultures has convinced us of the necessity of vaseline layers, glass ring, and of a nutritive liquid composed according to the above given specification.

In what concerns the necessity of damp plastic membranes, very numerous experiments were conducted on various surfaces: glass, cellophane, dry plastic membranes on glass, which convinced us of the irregularity and mostly of the insufficience and the lack of vigor of the obtained proliferation.

A control experiment demonstrated in a definite way the preference of epithelium for such plastic membranes. In our Petri dishes a series of cultures were conducted in the following manner: voluminous nutritive drops were disposed in rings drawn on the glass with hot vaseline. Explants, introduced into these drops, deposited themselves on the glass; at this moment minute drops of Formvar solution, deposited on the surface of the liquid drops immediately formed small plastic membranes. The excell liquid was drawn off in such a manner as to leave the tissuelar fragments in contact with the glass on the one hand and the plastic membrane on the other, in an atmosphere of nutritive liquid. The results of this experiment were conclusive: the epithelium always proliferated against the covered membrane and never on the glass surface. As a matter of fact growth was extremely mediocre.

Many authors have studied the mechanism of epithelial proliferation on unicellular membranes. In general one admits a propagation by amoeboid movements when the suffaces are adequate. We feel that hard surfaces (glass, mica, dry plastic membranes, cellophane) are less well suited to proliferation than soft and less shiny surfaces (plasma, damp membrane). Observation of our cultures has demonstrated that the epithelial cells

have a very definite tendency to follow the minute folds which might form here and there on plastic membranes; it is therefore conceivable, as this membrane is extremely fine, that the cellular movement itself might cause small folds which would be favorable to the extension of the epithelial membrane. Microfilm would clarify this question.

Applications

The histological study of virus action on various epitheliums, in particular the study of formation of inclusions, might profit from this technique allowing for rapid procurement of epithelial membrane devoid of cumbersome plasmatic support.

The study of virus variations, caused by the tissues also necessitates epithelial pure cultures, which serve as biological tests allowing to objectivate the alterations of tissuelar affinity. Extremely diverse epithelial parenchymes must therefore be cultivated for this end; certain preliminary experiments with chicken kidney tissue and adult mouse pulmonary tissue have shown us that with these tissues plastic membranes also allow an excellent epithelial culture.

It is interesting to note that performed epithelial membranes are exquisitely sensitive to the action of chemical products and to virus suspension (Robinow). We have been able to show that the concentration of borate of phenyl-mercury, product used to bacteriologically sterilize virus suspensions, is insufficient to stop tissue proliferation, has an extremely violent action, destructive, on preformed membranes. Thus everything seems to indicate that the virus test conducted on preformed membranes would be more sensitive than a test by virus action on simple proliferation on a culture tissue (Huang).

The epithelial membranes steming from parenchymes and from animal judicously chosen would perhaps serve to the clinical diagnostic of virus infections (Sanders), eventually in combination with specific immune serums.

It is not impossible that in certain fields pure epithelial cultures, could replace laboratory animals, on the condition that they can be obtained rapidly and in large quantities.

Conclusions

Humid plastic membranes form a particularly favorable surface for the rapid development of the epithelial membranes.

Pure epithelial cultures may thus be obtained without transplanting and without plasma within three days.

These cultures, devoid of explant, may be maintained in a vigorous state of proliferation by nutritive bathing.

The technique allows the study of various actions (virus, physico-chemical factors) on epithelial preformed membranes, with a minimum of work.

The obtained preparations, being devoid of plasma, may be stained and observed under immersion as smears. Certain applications are considered.

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by J. Stancioff